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A UV PROTECTIVE COMPOUND FROM *GLOMERELLA CINGULATA*—A MYCOSPORINE

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Key Word Index—*Glomerella cingulata*; Pyrenomycetes; mycosporine; structure; HPLC.

Abstract—The isolation and structure of a biologically important mycosporine are described. The use of Sephadex ion-exchange chromatography and HPLC provides a convenient method for the isolation of mycosporines.

INTRODUCTION

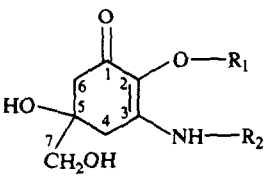
In the course of a study on the survival of conidia of *Glomerella cingulata* (Stoneman) Spaulding and Schrenk on the surfaces of apples exposed to solar radiation, Brook [1] found that the conidia were surrounded by a layer of mucilage which contained a substance with maximum absorbance at *ca* 310 nm. He also showed that the substance increased the survival rate of dark-grown *G. cingulata* conidia when these were exposed to middle wavelength UV radiation. The mucilage surrounding the conidia, produced in the absence of UV radiation, does not contain the UV absorbing material. A class of compounds which have maximum absorbance at around 310 nm and which have the substituted cyclohexenone system shown in structures **2–4**, has been previously isolated from fungi and algae [2–4]. These compounds have been given the generic name 'mycosporine' [5]. In this report, we describe the isolation and structure of the UV absorbing substance from *G. cingulata*, to which we have assigned the name mycosporine-glu (**1**).

RESULTS AND DISCUSSION

The new mycosporine could not be extracted from the conidia–mucilage mass with water. Once freed from the mass with ethanol however, it was readily soluble in water. The amino acid nature of the compound was established during preliminary experiments with a crude extract, using TLC and thin layer electrophoresis (TLE). Advantage was then taken of the amino acid properties in the purification steps. Partition between water and *n*-BuOH removed most of the organic solvent soluble material, while the bulk of the water soluble contaminants was removed using a modification of the Sephadex ion-exchange procedure described by Redgwell [6]. In contrast to the common amino acids, mycosporine-glu (**1**) was eluted off SP-Sephadex (H^+ form) with water. This was probably a consequence of the substituent on the amino group. Semi-prep. HPLC was used for the final purification. We found that Zorbax-ODS was superior to μBondpak C-18 packing (Waters Associates, U.S.A.) for this separation. A higher sample loading (0.1 mg) and increased capacity factor were obtained. Attempts to increase the rather low capacity factor, by using ion-pairing reagents were unsuccessful. After the completion of this work we were able to try the purification on a Radial Pak C-18 (10 μm) column (100 × 8 mm i.d.) from Waters Associates. Under the same HPLC conditions, this column could easily handle 5 mg injections. TLC was not suitable for the purification of mycosporine-glu since it was slightly unstable on cellulose and silica gel. Decomposition was most noticeable when the absorbent layers were dry.

We were able to obtain mycosporine-glu only as a glass which readily absorbed moisture from the atmosphere. When chromatographed on an analytical Zorbax-ODS column the final product gave a single peak with both the refractive index and absorbance detectors. Although mycosporine-glu is slightly unstable on cellulose and Si gel, we were able to confirm the homogeneity of the HPLC product by the 2-D TLC and TLE techniques in which the plates were developed in both directions with the same system. The plates were visualized with the less specific iodine vapour as well as ninhydrin.

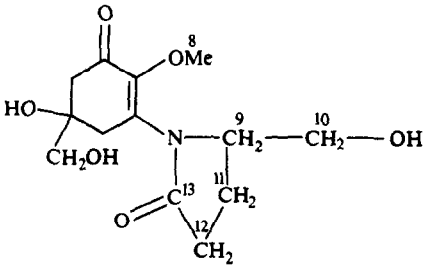
FDMS of mycosporine-glu gave a $[M]^+$ peak at *m/z* 317. With increasing emitter current (hence temperature) peaks appeared at *m/z* 299 $[M-18]^+$, 273 $[M-44]^+$ and 225 $[M-18-44]^+$. With increasing temperature the *m/z* 317 and 273 peaks rapidly lost intensity. At yet higher temperatures still, these peaks completely disappeared and an intense peak appeared at *m/z* 281. This peak was presumably due to the same ion as the *m/z* 281 peak in the EIMS spectrum obtained by the 'in-beam' technique [7]. The only other intense peak in the EIMS spectrum was the



1 R_1 = —^{8}Me R_2 = —^{9}CH(—^{11}CH$_2$—^{12}CH$_2$—^{13}COOH)—^{10}COOH

2 R_1 = —^{8}Me R_2 = —^{9}CH$_2$—^{10}COOH

3 R_1 = —H R_2 = —^{9}CH(—^{11}CH$_2$—^{12}CH$_2$—^{13}CONH$_2$)—^{10}COOH



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base peak at *m/z* 236. Mass measurement established that the *m/z* 281 and 236 peaks were due to $C_{13}H_{15}NO_6$ and $C_{12}H_{14}NO_4$ ions. The loss of two molecules of water to give the *m/z* 281 ion was probably a thermal elimination since it was only formed at high emitter current with FDMS. When the conventional probe method was used the spectrum obtained did not show any diagnostically useful features.

The ^{13}C NMR and ^{1}H NMR data were consistent with structure 1 and compared well with the assignments given for mycosporine-gly (2) [3] and mycosporine-2 (4) [8] for which data determined in D_2O were available. The AB quartets due to the C-4 and C-6 protons were not resolved at 200 MHz. Mycosporine-gly (2) gave glycine on heating to 80° in water [3]. Similarly, normycosporine glutamine (3) was reported to undergo facile hydrolysis to give glutamine, although the exact conditions were not given [2]. The present mycosporine (1), despite its instability when subjected to TLE and TLC, required several hours of heating in a boiling water bath for complete hydrolysis of the amino acid moiety. The presence of glutamic acid in the hydrolysis mixture was determined using the combined TLE and TLC method of Bieleski and Turner [9]. A check for glutamine and pyroglutamic acid proved negative.

The amount of pure mycosporine-glu available did not permit characterization of the cyclohexenone derivative which should also be produced by the hydrolysis reaction [2, 3].

Ito and Hirata [3] methylated the carboxyl group in mycosporine-gly (2) with diazomethane. Mycosporine-glu (1) gave multiple products when treated with an ethereal solution of diazomethane in the presence of methanol.

Mycosporines were originally associated with sporulation of fungi [10] and there have been suggestions that they may have a protective role [3]. However, mycosporine-glu is the first mycosporine that has been shown to have a definite biological role [1].

EXPERIMENTAL

EtOH extracts containing mycosporine-glu were kindly supplied by Dr P. J. Brook [1].

Chromatographic methods. TLC (mix cellulose–Si gel) with MeCOEt–C_5H_5N–H_2O–HOAC (70:15:15:2) and *n*-PrOH–H_2O–*n*-PrOAc–HOAc–C_5H_5N (120:60:20:4:1) and TLE was according to Bieleski and Turner [9] except that the inert liquid coolant was replaced by a cooled Al block underneath the TLE plate. The pH 7.9 buffer was 0.15 M NH_4HCO_3. Sephadex QAE-A-25 was pre-conditioned for 2 days in 0.5 M $KHCO_3$ followed by washing with 0.5 M NH_4HCO_3 (×4), 0.05 M NH_4HCO_3, and H_2O until neutral. Sephadex-SP-C25 was prepared as described by Redgwell [6] except that 0.5 M $HCOONH_4$ was used instead of $(NH_4)_2SO_4$. HPLC was carried out using 0.05 M NH_4HCO_3 at 0.5 ml/min on a commercial Zorbax-ODS column (250 × 4.6 mm) (Dupont, U.S.A.). For prep. HPLC, a column (300 × 3 mm) packed with 10 μm Zorbax-ODS was used. RI and UV (313 nm) detectors were used in series.

Purification of mycosporine-glu (3-glutamo-5-hydroxy-5-hydroxymethyl-2-methoxyhex-2-enone). Rotary evaporation was carried out at 35–40°. The EtOH extract supplied was kept overnight at 4°, centrifuged at 13000 *g* for 20 min and

the supernatant poured off. The ppt was discarded. The supernatant was rotary evaporated to dryness, taken up in H_2O (100 ml) and extracted with *n*-BuOH (3 × 100 ml). The aq. layer was rotary evaporated to dryness and dried over conc H_2SO_4 at < 0.01 torr to yield 2.9 g residue. This material was taken up in H_2O (20 ml) of which 10 ml were applied to the top of a Sephadex QAE (HCO_3^-) column (305 × 20 mm). The column was eluted with H_2O (130 ml) followed by a linear concn gradient going from H_2O (100 ml) through to 0.2 M NH_4HCO_3 (100 ml). Mycosporine-glu started to elute after 135 ml had been collected. A further 80 ml 0.2 M NH_4HCO_3 was passed down the column to completely elute the mycosporine. The second 10 ml of the crude extract was similarly chromatographed. The fractions absorbing at 311 nm were combined, rotary evaporated to *ca* 0.5 ml, kept at room temp. overnight to decompose the remaining NH_4HCO_3 and then taken to dryness. The residue was taken up in H_2O (1 ml) and chromatographed on a Sephadex SP-C25 (H^+) column (150 × 12 mm) using H_2O. The fraction with λ_{max} 311 nm was eluted in 8 ml, commencing after 15 ml had been collected. Evaporation to dryness gave 78.7 mg crude mycosporine-glu.

The crude material in 0.8 ml 0.05 M NH_4HCO_3 was subjected to prep. HPLC. Injections containing *ca* 10 mg of material were used. The peak corresponding to mycosporine-glu was collected in five fractions on a time basis. All five fractions required further purification. They were all rotary evaporated to *ca* 0.1 ml, kept overnight at room temp. and evaporated to dryness. The residues were again wetted with H_2O, kept overnight and evaporated to dryness since it was important to completely remove the NH_4HCO_3 to avoid getting extraneous peaks when using the RI detector. The residue in each fraction was taken up in 0.05 M NH_4HCO_4 and re-chromatographed by HPLC. Sample size per injection was kept to a maximum of 0.1 mg. Again the mycosporine-glu peak was collected in five fractions. Fractions 4 and 5, homogeneous by analytical HPLC, 2-D TLE and 2-D TLC were combined. The NH_4HCO_3 was decomposed by rotary evaporation to 0.1 ml and standing overnight, to give 7.6 mg of a glass after drying for 24 hr at < 0.001 torr. Fraction 3 (2.8 mg) was slightly impure.

Mycosporine-glu (**1**): H_2O λ_{max} nm (log ϵ): 311 (4.32); EIMS (in-beam) 20 eV, *m/z* (rel. int.): 281 $[M-2H_2O]^+$ (78), 236 $[281-45]^+$ (100); EIMS (in-beam, high resolution) 70 eV, *m/z*: 281.0893 ($C_{13}H_{15}NO_6$ requires 281.0899), 236.0908 ($C_{12}H_{14}NO_4$ requires 236.0922); FDMS *m/z* (rel. int.): 317 $[M]^+$ (100), 299 $[M-H_2O]^+$ (52), 273 $[M-44]^+$ (62), 255 $[299-44]^+$ (31); ^{13}C NMR (50.29 MHz, D_2O): δ 30.9 (*t*, C-11), 33.9 (*t*, C-12), 35.6 (*t*, C-4), 45.6 (*t*, C-6), 60.2 (*d*, C-9), 61.9 (*q*, C-8), 70.1 (*t*, C-7), 74.6 (*s*, C-5), 134 (*s*, C-2), 160.3 (*s*, C-3), 179.9, 180.5, 188.4 (all *s*, C-1, C-10, C-13); ^1H NMR (400 MHz, D_2O): δ 2.09 (1H, *m*, C-11), 2.17 (1H, *m*, C-11), 2.38 and 2.65 (2H, AB*q*, $J_{4\alpha,4\beta}$ = 17 Hz, C-4), 2.39 (2H, *t*, $J_{11,12}$ = 7.5 Hz, C-12), 2.68 and 2.72 (2H, AB*q*, $J_{6\alpha,6\beta}$ = 17.5 Hz, C-6), 3.51 (2H, *s*, C-7), 3.59 (3H, *s*, C-8), 4.07 (1H, *m*, C-9). HPLC retention vol.: 3.25 ml.

Hydrolysis of mycosporine-glu (**1**). Mycosporine-glu (300 μg) in H_2O (150 μl) was sealed under N_2 in a small Pyrex tube and heated in a boiling water bath for 5 hr. The presence of glutamic acid in the hydrolysis mixture was determined using combined TLE and TLC.

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